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(54) Shaped detergent composition comprising mutant subtilisin

(57) A shaped detergent composition comprises a detergent active, water and a mutant subtilisin enzyme in which the amino acids in positions 185 and 222 in the native enzyme have been replaced with different amino acids (preferably with glutamic acid and alanine, respectively).

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DETERGENT COMPOSITION

The present invention relates to detergent compositions in the form of bars, tablets, sticks and the like for direct application to fabrics, hard surfaces or any other surface. In particular, it relates to soap or soap/synthetic compositions in bar form which bars also include protease enzymes.

10 It is known to incorporate protease enzymes into soap bars. However, enzymes tend to be unstable, that is they lose activity, in such bars. According to British Patent 265 024 this disadvantage may be overcome by dehydrating the enzyme and the soap bar to a water content of 10% or
15 less.

As an alternative, and in order to produce bars which contain greater than 10% of water, the use of stabilising agents for protease enzymes have been proposed. Many
20 stabilising agents cannot readily be used in bars or other similar shaped solid products because of the adverse effect they have on the structure and firmness of the product.

25 According to British Patent 2 186 883, it is proposed to use a mixture of a boron compound, a polyol, an organic acid or its alkali metal salt and an alkali metal salt of an inorganic acid which is not a boron compound as a stabilising system for protease enzymes, such as Savinase,
30 in soap bars.

It is also known to use mutant subtilisin proteases which have been modified by substitution at an amino acid site. US 4 760 025 (Genencor), for example, claims subtilisin mutants with amino acid substitutions at amino acid sites
35 32, 155, 104, 222, 166, 64, 33, 169, 189, 217 or 157.

A mutant protease whereby methionine at position 222 has been replaced by alanine, is shown to have an improved oxidation stability in the presence of a bleaching agent.

5 We have now found it is possible to prepare detergent compositions in bar or other shaped solid form with a relatively high level of water, typically 10 to 30% by weight, with mutant subtilisin enzymes which have been modified at positions 195 and 222 in the protease. Such
10 compositions, particularly in the form of soap bars, have improved enzyme storage stability compared to soap bars comprising other commonly used protease enzymes such as Savinase, even in the absence of a bleaching agent. Furthermore, as a result of this increased enzyme
15 stability, there are improvements in the performance of the bars.

Thus, accordingly the present invention provides a detergent composition in shaped solid form comprising a detergent active, water, and a mutant subtilisin enzyme in which the amino acid sequence has been altered at positions 195 and 222 by substitution with another amino acid.

25 Preferably, the detergent composition will also contain a stabilising system comprising a boron compound and a polyol.

The stabilising system may also additionally contain
30 at least one further component selected from

- i) an organic acid, an alkali metal salt of an organic acid, an inorganic acid and mixtures thereof; and
- 35 ii) an alkali metal salt of an inorganic acid not being a boron compound.

An advantage of the compositions according to the invention is that they can be stored for much longer periods of time, even at conditions of high temperature and relative humidity, without any significant loss in enzyme activity, than similar compositions previously proposed. This is important since such detergent compositions in bar or other shaped solid form are typically used in countries where such conditions prevail.

Boron compounds suitable for the stabilising system include boric acid, boric oxide and alkali metal borates and, in particular, borax. The polyol may be an aliphatic polyol with 2 to 6 carbon atoms and 2 to 6 hydroxyl groups, especially propylene glycol, glycerol and sorbitol. The organic acid may be an aliphatic mono-, di-, or tricarboxylic acid, and in particular formic acid, succinic acid, adipic acid, glutaric acid, citric acid or commercially available mixtures thereof, for example Sokalan DCS (Trade Mark), and soap acids such as tallow, coconut, palm kernel and babassu fatty acids. Suitable alkali metal salts of organic acids include sodium formate, sodium citrate and sodium propionate. A suitable inorganic acid is phosphoric acid. Alkali metal salts of inorganic acids, not being a boron compound, include sodium sulphate, sodium chloride, sodium carbonate, sodium bicarbonate and sodium phosphate.

The boron compound is preferably present in an amount of 0.1 to 10%, most preferably 0.5 to 5%; the polyol preferably 0.1 to 15%, most preferably 0.5 to 10%; the organic acid or salt thereof or inorganic acid in amount of 0.1 to 10%, preferably 0.5 to 5%; and the alkali metal salt of the inorganic acid which is not a boron compound, in an amount 0.1 to 5%, preferably 0.25 to 2.5%; where all percentages are by weight of the composition.

Preferably bars in accordance with this invention also comprise:

- 5 i) 25 to 80%, most preferably 25 to 70%, by weight of detergent active which is soap or a mixture of soap and synthetic detergent active, reckoned as anhydrous;
 - 10 ii) 0 to 35% and, most preferably, 10 to 30% by weight of water;
 - 15 iii) 0 to 35% and, most preferably, 0.1 to 30% by weight filler.
- When a synthetic detergent active is included in the bar it will preferably be present at a level between 0.1 and 10%, most preferably 0.5 to 5% by weight based on the total level of detergent active.
- Fatty acid soaps suitable for use herein can be obtained from natural sources such as, for instance, plant or animal esters (e.g. palm oil, coconut oil, babassu oil, soybean oil, castor oil, tallow, whale or fish oils, grease, lard and mixtures thereof). The fatty acid soaps can also be synthetically prepared (e.g. by the oxidation of petroleum, or by the hydrogenation of carbon monoxide by the Fischer-Tropsch process). Resin acids, such as those present in tall oil, may be used. Naphthenic acids are also suitable.
- Tallow fatty acids can be derived from various animal sources and generally comprise about 1% to 8% myristic acid, about 21% to 32% palmitic acid, about 14% to 31% stearic acid, about 0% to 4% palmitoleic acid, about 36% to 50% oleic acid and about 0% to 5% linoleic acid. A typical distribution is 2.5% myristic acid, 29% palmitic

acid, 23% stearic acid, 2% palmitoleic acid, 41.5% oleic acid, and 3% linoleic acid.

5 Coconut oil refers to fatty acid mixtures having an approximate carbon chain length distribution of: 8% C₈, 7% C₁₀, 48% C₁₂, 17% C₁₄, 8% C₁₆, 2% C₁₈, 7% oleic and 2% linoleic acids (the first six fatty acids listed being saturated). Other sources having similar carbon chain length distributions, such as palm kernel oil and babassu 10 kernel oil, are included within the term coconut oil.

Coconut oil fatty acids ordinarily have a sufficiently low content of unsaturated fatty acids to have satisfactory keeping qualities without further treatment. Generally, however, fatty acids are hydrogenated to decrease the 15 amount of unsaturation (especially polyunsaturation) of the fatty acid mixture.

20 Babassu oil refers to fatty acid mixtures having an approximate carbon chain length distribution of: 7.8% C₈, 6.5% C₁₀, 44% C₁₂, 15.2% C₁₄, 7.9% C₁₆, 3.2% C₁₈, 12% C_{18.1}, 2.3% C_{18.2}. (The last two being mono and double unsaturated respectively)

25 It is also possible to include small amounts of non-soap detergent active in compositions of the invention such as branched alkyl benzene sulphonates, linear alkyl benzene sulphonates and nonionic ethoxylated alcohols.

30 The filler is preferably selected from kaolin, bentonite, silicate, talc, sodium sulphate and sodium carbonate.

35 The mutant subtilisin enzymes used in the composition of the invention are disclosed in WO-A-89/06279 (Novo/Nordisk). They differ from the native subtilisin enzyme in that they contain a different amino acid at positions 195 and 222. The native enzyme contains a

glycine residue at position 195 and a methionine at position 222. A particularly preferred mutant enzyme is one which contains a glutamic acid residue at position 195 and an alanine residue at position 222. A commercially available material with the aforementioned mutations is Durazym (ex Novo/Nordisk). The enzyme may also be mutated at sites other than those specified hereinbefore.

In general, the amount of mutant subtilisin enzyme included in the composition of the invention is such that it corresponds with a proteolytic activity of 0.1 to 100 GU/mg based on the composition, preferably 0.5 to 20GU/mg, most preferably 1.0 to 10GU/mg, where GU/mg is glycine unit per milligram.

A glycine unit is defined as the enzyme activity which under standard conditions, during a 15 minute incubation at 40°C with N-Acetyl casein as substrate, produces an amount of terminal NH₂-groups equivalent to 1 microgramme/ml of glycine.

The compositions of the invention may contain other proteolytic enzymes in addition to the mutant subtilisin enzyme hereinbefore defined. Further subtilisin proteases can be of vegetable, animal or microorganism origin. Preferably it is of the latter origin, which includes yeast, fungi, moulds and bacteria. Particularly preferred are bacterial subtilisin type proteases, obtained from e.g. particular strains of *B. subtilis* and *B. licheniformis*. Examples of suitable commercially available proteases are Alcalase, Savinase, Esperase, all of Novo/Nordisk A/S; Maxatase and Maxacal of Gist-Brocades; Kazusase of Showa Denko; Subtilisin BPN' proteases and so on.

The compositions of the invention may also contain enzymes

such as lipases, for example Lipolase (Trade Name ex Novo/Nordisk), a lipase extracted from *Rumicolum Lanuginosa*; amylases, a commercially available material is sold under the Trade Mark Termamyl ex Novo/Nordisk; and cellulases, a commercially available material is sold as Celluzyme (Trade Mark) ex Novo/Nordisk.

Enzymes may be incorporated in the detergent composition of the invention in the form of a concentrated aqueous liquid or a slurry.

In addition to the components described above the detergent bars of the present invention may contain a wide variety of optional materials. For example builders such as water-soluble phosphate salts, water soluble carbonates and organic builders may be added. Other ingredients such as starch, sodium carboxymethylcellulose, colouring materials, fluorescers, opacifiers, germicides, perfumes, preservatives for example ethylene diamine tetra-acetic acid (EDTA), ethane 1-hydroxy-1,1'-diphosphonic acid (EHDP), formaldehyde and sodium hypochlorite, sucrose and sorbitol may also be included.

Bars according to the invention may be prepared on conventional equipment and in a conventional manner. The mutant subtilisin enzyme, any other additional enzyme and the components of the stabilising system may be added at any stage during the manufacturing process. Preferably the stabilising components are added before the enzyme. The resulting mixture of components may then be plodded in conventional manner and, if necessary, stamped, left to age wrapped.

The invention will now be described further and exemplified in the following examples in which percentages are by weight.

Example I

Hard soap bars were produced from conventional soap (82% tallow, 18% coconut oil) produced by pan saponification. The soap was neutralised with 0.15% orthophosphoric acid
5 and then mixed for approximately 20 minutes at a temperature of 80°C. Thereafter, the stabilising system was added and mixing continued for a further hour while the temperature was maintained at 80°C. The water content of the resulting mixture was reduced by vacuum drying to
10 the desired moisture content and the enzyme added. The resulting mixture was then plodded on a soap production line in a conventional manner.

In each case, the water content of the bars and the total
15 fatty matter content were determined and found to be in the range 17-19% and 65-70% by weight respectively. The bars were wrapped individually in glycine paper and/or polyethylene bags and stored at 30°C. The residual enzyme activity of bars was measured at intervals during storage
20 using a 5101 Scalar Aautoanalyser.

In each case, part of the bar was dissolved to form a weak aqueous solution. The enzyme activity was determined by photometric detection of a complex formed from reaction of
25 the enzyme in the solution with a protease substrate. The results are presented below.

Composition		Enzyme activity %			
		Storage time (days)	14	27	41
30 i) enzyme containing soap bar with sodium formate(2%), borax(2%), propylene glycol(2%), sodium sulphate (1%). enzyme:-					
35 a) Savinase® 8GU/mg (moisture 17.5%, TPM® 68.7%)			82	83	79

	b)	Durazym**	8GU/mg	100	100	100	
			(moisture 17.4%, TFM ⁱ 65.4%)				
5	ii)	enzyme containing soap bar with sodium formate(2%), borax (2%), propylene glycol(2%), sucrose (5%), sodium sulphate (1%). enzyme:-					
10	a)	Savinase	8GU/mg	89	92	85	
		(moisture 17.4%, TFM 64.1%)					
b)	Durazym	8GU/mg	100	100	100		
		(moisture 17.0 %, TFM 65.4%)					
15	iii)	enzyme containing soap bar with borax(2%), propylene glycol(2%), sucrose (5%), sodium sulphate (1%) enzyme:-					
a)	Savinase	8GU/mg	87	84	75		
		(moisture 18.7%, TFM ⁱ 64.8%)					
20	b)	Durazym	8GU/mg	100	100	100	
		(moisture 18.8%, TFM ⁱ 65.6%)					
*	Savinase 8.0L (ex Novo/Nordisk A/S)						
**	Durazym 16.0L (ex Novo/Nordisk A/S), a mutant subtilisin protease containing a glutamic residue at position 195 and an alanine residue at position 222.						
25	ⁱ	Total fatty matter.					

The results show a bar containing the mutant subtilisin enzyme Durazym is much more stable, that is residual enzyme activity of the bar after storage is much higher, than a bar containing the enzyme Savinase.

Example II

In this example the variation of residual enzyme activity (enzyme stability) with level of the stabiliser system was examined.

5. Soap bars of compositions A and B were prepared according to the procedure outlined in Example I.

	Composition	A	B
		% by weight	
10	Citric acid	1	0.5
	Sodium sulphate	1	0.5
	Sodium Formate	2	1
	Glycerol	2	1
	Borax	2	1
15	Durazym** 5 GU/mg of the bar composition	0.3	0.3
	Soap base (80% tallow, 20% coconut oil)		
	** Durazym 16.0L	(ex Novo/Nordisk A/S)	

20. The bars were wrapped individually in glycine paper and stored at 37°C and a relative humidity of 70. The residual enzyme activity of bars was measured periodically during storage.

25. The results are presented below.

		Residual Enzyme Activity/%	
Composition		A	B
Storage Time/Days			
5	0	100	100
	7	~	92
	21	~	82
	35	~	80
	43	~	78
	49	~	76
10	50	84	~
	57	89	~
	64	82	~
	70	~	68
	71	80	~
15	91	~	66
	92	84	~
	105	~	59
	106	78	~
	177	76	~

20

The results show that even when the amount of the stabilising system is reduced the enzyme activity remains at an acceptable level.

Example III

In this example the residual enzyme activity (enzyme stability) of bars prepared using slurries of the mutant subtilisin enzyme Durazym and Savinase were compared.

5

Soap bars of compositions C and D were prepared according to the procedure outlined in Example I.

	Composition	C	D
		% by weight	
10	Citric acid	1	1
	Sodium sulphate	1	1
	Sodium Formate	2	2
	Glycerol	2	2
15	Borax	2	2
	Enzyme		
	Durazym slurry*	0.3	-
	Savinase slurry	-	0.3
	Soap base (80% tallow, 20% coconut oil)		

* Durazym 16.05L (Novo/Nordisk A/S)

The bars were wrapped individually in glycine paper and stored at 37°C and a relative humidity of 70%. The residual enzyme activity of bars was measured periodically during storage.

The results are presented below.

		Residual Enzyme Activity/%	
30	Composition	C	D
	Storage Time/Days		
	0	100	100
	7	-	80
	14	100	60
35	35	98	40
	70	89	37

The results show a bar containing the mutant subtilisin enzyme Durazym has a higher enzyme stability than a corresponding bar containing the enzyme Savinase.

5 Example IV

In this example, the cleaning performance of bars containing Savinase and Durazym liquid enzymes were compared.

- 10 Soap bars of compositions E and F were prepared according to the procedure outlined in Example I.

	Composition	E	F
		% by weight	
15	Coconut fatty acid	1	1
	Sodium Sulphate	1	1
	Sodium Formate	1	1
	Glycerol	2	2
	Borax	2	2
20	Enzyme Durazym Liquid** (5 GU/mg of the bar composition)	0.12	-
	Savinase Liquid*** (5 GU/mg of the bar composition)	-	0.12
25			

**Durazym 16.0L (ex Novo/Nordisk A/S)

***Savinase 16.0L (ex Novo/Nordisk A/S)

- 30 After manufacture, but before testing, the bars were stored at 4°C to prevent enzyme degradation. Bars were stored for various lengths of time before testing. Thereafter, bars of compositions E and F were used to wash standard test cloths using a standardised procedure based on a handwashing procedure commonly used with laundry bars.

The test cloth was immersed in a quantity of deionised water. The wetted test cloth was squeezed out, reimmersed and squeezed out a second time, and then treated with a bar. The bar was rubbed by hand on the cloth. The bar was then set aside and the treated test cloth was immersed in deionised water, test cloth to liquor ratio of 2.8g/L which is equivalent to standard wash conditions, and rubbed 30 times. The test cloth was rinsed in clean water and dried.

10

The reflectance, at 460nm, of the test cloth was measured before and after washing using a Micromatch Reflectance Spectrometer. AR is the change in reflectance.

15

The procedure outlined above was repeated four times for each bar composition at each storage time, and the results presented below represent an average value.

20

Storage Time/Days

ΔR_{460}

E

F

25

		ΔR_{460}	
	Storage Time/Days	E	F
	0	17.24	19.45
	6	16.87	17.07
	8	16.80	16.73
	10	16.77	16.38
	12	16.74	16.04
	20	16.60	14.68
	30	16.43	12.97

30

The results show the increased stability of Durazym containing bars, gives rise to an overall performance benefit after a storage time of only 6-8 days.

35

In the following examples soap bars of compositions G, H, I and J were all prepared according to the procedure outlined in Example I.

Example V

In this example the residual enzyme activity (enzyme stability) of a bar prepared with coconut fatty acid, but in the absence of sodium formate, was examined.

5

	Composition	G
		\$ by weight
	Coconut fatty acid	1
	Sodium Sulphate	1
10	Sodium Formate	-
	Glycerol	2
	Borax	2
	Durazym † (5 GU/mg of the bar composition)	0.3
15	Soap base (80% tallow, 20% coconut oil)	

† Durazym 16.0L (ex NOVO/Nordisk A/S)

20 The bar was wrapped in glycine paper and stored at 37°C and a relative humidity of 70. The residual enzyme activity of the bar was measured periodically during storage.

25 The results are presented below.

	Composition	Residual Enzyme Activity/%
	Storage Time/Days	
	0	100
30	13	100
	21	100
	40	83
	80	81

Example VI

In this example residual enzyme activity (enzyme stability) of a bar prepared with phosphoric acid was examined.

5

	Composition	H
		% by weight
	Phosphoric Acid	0.15
	Sodium Sulphate	1
10	Sodium Formate	2
	Propylene Glycol	2
	Borax	2
	Durazym † (5 GU/mg of the bar composition)	0.3
15	Soap base (80% tallow, 20% coconut oil)	

† Durazym 16.0L (ex NOVO/Nordisk A/S)

20

The bar was wrapped in glycine paper and stored at 28°C and a relative humidity of 70. The residual activity of the bar was measured periodically during storage.

25

	Composition	Residual Enzyme Activity/%
	Storage Time/Days	
	0	100
	14	100
30	27	100
	41	100

Example VII

In this example the residual enzyme activity (enzyme stability) of bars prepared with and without Sodium Formate was examined.

	Composition	I	J
		% by weight	
	Citric acid	1	1
	Glycerol	6	6
10	Sodium Formate	2	-
	Borax	2	2
	Durazym ‡ (5 GU/mg of the bar composition	0.3	0.3
	Soap base (80% tallow, 15 20% coconut oil)		

‡ Durazym 16.0L (ex NOVO/Nordisk A/S)

The bars were wrapped individually in glycine paper and stored at 37°C and a relative humidity of 70. The residual enzyme activity of the bars were measured periodically during storage.

The results are presented below.

	Composition	Residual Enzyme Activity/%	
		I	J
	Storage Time/Days		
	0	100	100
	10	100	100
30	24	100	100
	30	100	100
	71	-	89
	76	-	-
	92	-	85
35	107	82	-

The results demonstrate that even in the absence of sodium formate good residual enzyme activity is maintained.

Claims

1. A detergent composition in shaped solid form comprising a detergent active, water, and a mutant subtilisin enzyme in which the amino acid sequence has been altered at positions 195 and 222 by substitution with another amino acid.
5
2. A detergent composition according to Claim 1 further comprising a stabilising system for the enzyme, the stabilising system comprising a boron compound and a polyol.
10
3. A detergent composition according to Claim 2 wherein the stabilising system further comprises at least one component selected from
15
i) an organic acid, an alkali metal salt of an organic acid, an inorganic acid and mixtures thereof; and
20
ii) an alkali metal salt of an inorganic acid not being a boron compound.
25
4. A detergent composition according to Claim 1 wherein the enzyme contains a glutamic acid residue at position 195 and an alanine residue at position 222.
5. A detergent composition according to Claim 1 wherein the composition contains the enzyme in an amount such that it corresponds with a proteolytic activity of 0.1 to 100 GU/mg based on the composition.
30
6. A detergent composition according to Claim 1 comprising 25 to 80% by weight of soap or a mixture of soap and synthetic detergent active, 10 to 30% by
35

weight of water and 0.1 to 30% by weight of filler.

7. A detergent composition according to Claim 3 wherein
the boron compound is borax; the polyol is selected
from propylene glycol, glycerol and sorbitol; the
organic acid is selected from formic acid, succinic
acid, adipic acid, glutaric acid, citric acid, soap
acids and mixtures thereof; the alkali metal salt of
the organic acid is selected from sodium formate,
sodium citrate and sodium propionate; the inorganic
acid is phosphoric acid; and the alkali metal salt of
the inorganic acid is selected from sodium sulphate,
sodium chloride, sodium carbonate, sodium bicarbonate
and sodium phosphate.
- 15 8. A detergent composition according to Claim 3 wherein
the boron compound is present in an amount from 0.1
to 10% by weight; the polyol is present in an amount
from 0.1 to 15% by weight; the organic acid, salt
thereof, or inorganic acid is present in an amount
from 0.1 to 10% by weight; and the alkali metal salt
of the inorganic acid is present in an amount from
0.1 to 5% by weight of the composition.
- 20 25 9. Use of a detergent composition according to any
preceding claim to treat a stained fabric.

Relevant Technical Fields		Search Examiner C SHERRINGTON
(i) UK Cl (Ed.M)	CSD (DDA, DEX, DHA); C3H (HB7M)	
(ii) Int Cl (Ed.5)	C11D 3/386; C12N 9/50, 15/57	Date of completion of Search
Databases (see below) (i) UK Patent Office collections of GB, EP, WO and US patent specifications.		Documents considered relevant following a search in respect of Claims :- 1-9
(ii) ONLINE DATABASES: WPI, CLAIMS		

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| A: | Document indicating technological background and/or state of the art. | &: | Member of the same patent family; corresponding document. |

Category	Identity of document and relevant passages		Relevant to claim(s)
Y	GB 2186883 A	(UNILEVER PLC) - whole document	1-9
Y	EP 0405902 A1	(UNILEVER PLC) - whole document, especially Claim 1(a),(b),(e) to (h)	1
Y	WO 89/06279 A1	(NOVO INDUSTRI) - whole document, especially Claim 60	1
X,Y	WO 92/08778 A1	(NOVO NORDISK) - whole document, especially page 5, lines 26 to 27	X:1 Y:1-9
P,A	WO 93/08253 A1	(UNILEVER PLC) - whole document	1
Y	US 4760025	(GENECOR INC) - whole document especially column 11, lines 36 to 50; Example 16; Claims 1 to 3	1

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